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Delayed Chromosomal Instability Induced by DNA Damage

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DNA damage induced by ionizing radiation can result in gene mutation, gene amplification, chromosome rearrangements, cellular transformation, and cell death. Although many of these changes may be induced directly by the radiation, there is accumulating evidence for delayed genomic instability following X-ray exposure. We have investigated this phenomenon by studying delayed chromosomal instability in a hamster-human hybrid cell line by means of fluorescence in situ hybridization. We examined populations of metaphase cells several generations after expanding single-cell colonies that had survived 5 or 10 Gy of X rays. Delayed chromosomal instability, manifested as multiple rearrangements of human chromosome 4 in a background of hamster chromosomes, was observed in 29% of colonies surviving 5 Gy and in 62% of colonies surviving 10 Gy. A correlation of delayed chromosomal instability with delayed reproductive cell death, manifested as reduced plating efficiency in surviving clones, suggests a role for chromosome rearrangements in cytotoxicity. There were small differences in chromosome destabilization and plating efficiencies between cells irradiated with 5 or 10 Gy of X rays after a previous exposure to 10 Gy and cells irradiated only once. Cell clones showing delayed chromosomal instability had normal frequencies of sister chromatid exchange formation, indicating that at this cytogenetic endpoint the chromosomal instability was not apparent. The types of chromosomal rearrangements observed suggest that chromosome fusion, followed by bridge breakage and refusion, contributes to the observed delayed chromosomal instability.

Cancer is thought to be caused by a progressive series of genetic alterations in a limited number of specific genes: the so-called oncogenes and tumor suppressor genes. Each alteration, whether associated with initiation or progression, may be mediated through a gross chromosomal change and therefore has the potential to be cytogenetically visible (43). Indeed, more than 100 recurrent translocations have been described from available information on more than 14,000 neoplasms with karyotypic abnormalities (30). The study of chromosomal rearrangements, their causation, and their effect on cellular processes is therefore important in gaining a more complete understanding of carcinogenesis and cancer treatment.

An important feature of studies of DNA damage and repair is that exposure of cells to DNA-damaging agents, e.g., ionizing radiation, rapidly results in a dose-dependent increase in chromosomal breakage and gross structural chromosomal rearrangements (1). In general, cells showing chromosome deletions and/or asymmetric chromosome exchange formation, such as rings and polycentric chromosomes, die at the subsequent mitosis because of abortive cell division or loss of acentric chromosome fragments (1, 5). Consequently, international cytogenetic protocols for testing the chromosome-damaging effects of potential mutagens and carcinogens call for analysis of metaphase chromosomes from the first mitosis after exposure. Likewise, chromosome analysis of first-division metaphases is used to determine chromosome damage in individuals occupationally or accidentally exposed to potential DNA-damaging agents, as well as to provide estimates of potential genetic risk that govern human exposure levels. Damage-induced symmetric chromosomal rearrangements, e.g., reciprocal translocations, inversions, and insertions, on the other hand, are thought to persist stably within the cell and can be detected several decades after exposure (1, 5, 21).

In recent years, evidence has accumulated to suggest that genomic instability can manifest several generations after cellular exposure to physical or chemical DNA-damaging agents. Some examples are delayed reproductive cell death in cells surviving X irradiation (6–9, 32, 40), delayed mutation in cells exposed to the alkylating agent ethyl methanesulfonate (44) or to X irradiation (7, 32, 40), and delayed chromosomal instability in cells surviving exposure to ionizing radiation (20, 23, 26, 39). In all cases the cells initially survived exposure to the DNA damage and were capable of reproductive cell proliferation. The genomic instability was then manifested in the progeny of those surviving cells. To further investigate delayed chromosome instability following DNA damage, we have used fluorescence in situ hybridization to detect chromosomal rearrangements in a human-hamster somatic hybrid cell line several generations after exposure to ionizing radiation.

MATERIALS AND METHODS

Cell culture. The human-hamster somatic hybrid cell line GM10115, produced by fusing Chinese hamster ovary (CHO) cells (UCW56) with human leukocytes, was obtained from the Human Genetic Mutant Cell Repository (line HHW416; Institute for Medical Research, Camden, N.J.). The hybrid contains a single copy of human chromosome 4 in a background of 20 to 26 hamster chromosomes. Chromosome 4 of the hybrid shows a normal dose-response curve for aberration induction measured at the first mitosis after exposure to either X rays or restriction endonucleases (30a). Cells were maintained as a monolayer in Dulbecco's modified Eagle's medium supplemented with 4.5 g of glucose per liter, 10% fetal bovine serum, 100 U of penicillin per ml, 100 mg of streptomycin per ml, 2 mM L-glutamine, and 0.2 mM proline. Cells were cultured at 34°C in an atmosphere of 5%

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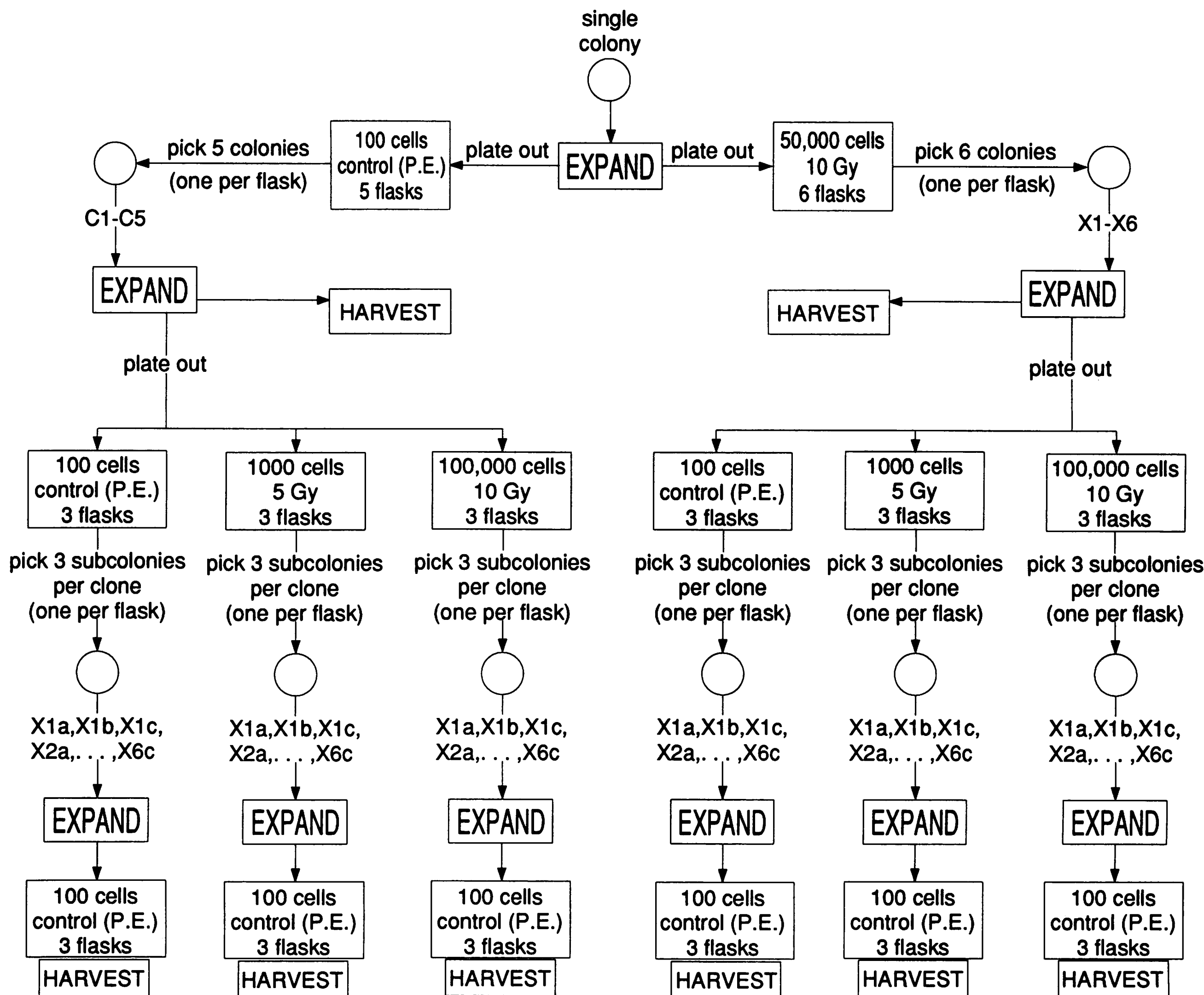


FIG. 1. Schematic diagram of the protocol used to investigate chromosomal instability in hamster-human hybrid GM10115 cells. The circles represent colonies of 60 to 70 cells originating from a single cell. The letters and numbers under the circles are the names given to the clones isolated.

CO_2 in air. One hundred cells were seeded into each of five 60-mm-diameter dishes and cultured to form single-cell colonies. One colony was chosen from each dish and expanded in two 75-cm^2 tissue culture flasks. One flask from each colony was analyzed for the presence of human chromosome 4. One subclone containing a single human chromosome 4 in >98% of the cells was then used in the following experiments.

Experimental protocol. The protocol is summarized diagrammatically in Fig. 1. Five 100-cell and six 50,000-cell samples from the expanded single-cell colony were seeded into 25-cm^2 tissue culture flasks. One hour later, flasks containing 50,000 cells were exposed to 10 Gy of X rays from a Philips RT100 X-ray machine (250-kV peak, 15 mA; half-value layer 1.0 mm Cu) at a dose rate of 2.5 Gy/min. These cells were cultured for 10 to 15 days to form colonies, after which time a single colony was chosen from each of the five unirradiated (control) flasks and from each of the six irradiated flasks. All colonies selected were of similar size (60 to 70 cells) to reduce possible X-ray-induced heritable damage that manifests as small colony size (41). The colonies remaining on the dish were then stained for 3 to 5 min with 0.25% crystal violet in 25% ethanol to determine plating efficiency (PE; number of colonies with >50 cells/number of cells plated) for the unirradiated cells and surviving fraction (number of colonies with >50 cells/number of cells plated × PE) for the irradiated cells.

The single-colony isolates were expanded, and 100, 1,000, or 100,000 exponentially growing cells were seeded into 25-cm^2 tissue culture flasks. Three flasks of cells were established at each population density for each of the five unirradiated and six irradiated single-colony isolates. The remaining unseeded cells from each of the original single-colony isolates were cultured overnight and processed the following day to determine chromosome rearrangements involving human chromosome 4. One hour after the initiation of culture, the flasks containing 1,000 cells were exposed to 5 Gy of X rays, and the flasks containing 100,000

cells were exposed to 10 Gy of X rays. Those flasks with 100 cells served as unirradiated controls and were used to determine PE. Both irradiated and unirradiated populations were cultured for 10 to 15 days to form colonies.

A single surviving colony of 60 to 70 cells was chosen from each of the three flasks exposed to 5 or 10 Gy of X rays, as well as from each of the three unirradiated flasks, and these were independently expanded into 25-cm^2 tissue culture flasks. The remaining colonies in the flasks were stained to determine PE or surviving fraction as appropriate. Exponentially growing cells from this second round of single-colony isolates were plated at 100 cells per flask into three flasks for each treatment group to determine PE, and the remaining cells were cultured overnight and processed the following day to determine chromosomal rearrangements involving human chromosome 4.

In one instance, when significant chromosomal instability was observed in the original single-colony isolate, 10 colonies were isolated from each of the three dishes containing the unirradiated cells. One colony isolate from each flask was treated as described above, and the remaining 27 colony isolates were expanded, analyzed cytogenetically to determine which were still exhibiting delayed chromosomal instability, and then stored frozen at −70°C. Some of these cells were used later to investigate the possible role of chromosome instability in sister chromatid exchange (SCE) formation.

Cytogenetic analysis. To obtain metaphase chromosomes, we added Colcemid (2×10^{-7} M final concentration) to exponentially growing cells for 2 to 3 h. Metaphase cells were collected after gentle shaking of the flasks to dislodge those cells in mitosis. Chromosome preparations were obtained by treating cells with 0.075 M KCl for 2 to 4 min, fixing them in methanol, washing them in methanol-acetic acid (3:1 [vol/vol]), and then dropping the cell suspension onto glass microscope slides. Slides were stored at −20°C.

Fluorescence in situ hybridization. A plasmid vector containing human chromosome 4-specific DNA sequences (pBS4) was kindly provided by J. Gray and D. Pinkle (University of California, San Francisco). The plasmid was amplified in *Escherichia coli* and purified with a Qiagen plasmid kit (Qiagen Co.). Purified plasmid was labeled (1 μg per reaction) with biotin by using a BioNick Kit (Bethesda Research Laboratories). Labeled DNA was separated from unincorporated nucleotides by using NucTrap Push Columns (Stratagene) and resuspended in 120 μl of nick translation-Push Column buffer.

The hybridization protocol and reagents were obtained from a chromosome in situ kit (Oncor). Briefly, slides were treated with RNase (100 μg/ml in 2× SSC [1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate]) for 1 h at 37°C and dehydrated in an iced ethanol series (70, 80, and 95% ethanol, 2 min per concentration). After drying, chromosomes were denatured in 70% formamide–2× SSC at 70°C for 2 min and dehydrated in an iced ethanol series (70, 80, 90, and 100% ethanol, 2 min per concentration). Thirty-five microliters of hybridization mix (50% formamide, 2× SSC, 10% dextran sulfate, 35 ng of labeled pBS4) was applied to slides under a glass coverslip (24 by 50 mm) and sealed with rubber cement. After overnight hybridization at 37°C in a humidified chamber, slides were washed in 50% formamide and 2× SSC at 37°C for 20 min and rinsed twice in 2× SSC at 37°C for 4 min each time. Slides were immersed in phosphate-buffered detergent (Oncor) and then treated at ambient temperature with Oncor blocking reagent for 5 min and incubated for 20 min with fluorescein-conjugated avidin. Slides were washed in three changes of phosphate-buffered detergent, and if necessary, the fluorescence signal was amplified by subsequent incubation with anti-avidin and fluorescein-conjugated avidin. In general, one round of amplification was sufficient. After fluorescein treatment, Antifade (Sigma) and propidium iodide were applied to slides under a glass coverslip. Metaphase chromosomes were analyzed on a Zeiss photomicroscope III equipped with the Epi-fluorescence condenser IIIRS and a standard fluorescein isothiocyanate filter set. Two hundred independent metaphase cells containing human chromosome 4 were analyzed (100 by each investigator), and the number of metaphase populations containing rearrangements involving chromosome 4 was determined.

SCE analysis in unstable populations. Three single-colony isolates with various degrees of instability, along with cells from the original expanded colony as a control, were cultured from stocks stored at −70°C (see above). Exponentially growing cells were cultured for two replication cycles (38 to 44 h) in 5-bromodeoxyuridine at a final concentration of 2×10^{-5} M. Metaphase cells were collected, and chromosome preparations were made as described above. Bromodeoxyuridine-substituted chromosomes were stained by a slight modification (31) of the fluorescence-plus-Giemsa technique of Perry and Wolff (36). Slides were stained with Hoechst 33258 for 20 min, mounted in Sorensen's buffer (pH 6.8), exposed to black light for 4 min, and then stained with 5% Giemsa for 20 min. Twenty-five second-division metaphase cells were scored for the yield of SCEs in each cell clone.

RESULTS

X-ray survival. Dose-dependent X-ray survival for the hamster-human cell hybrids closely resembled that of normal CHO cells (12). The average surviving fraction of cell hybrids irradiated with 5 Gy was 0.094 ± 0.016 (mean ± standard deviation), and that of cell hybrids irradiated with 10 Gy was 0.00127 ± 0.00039. These values were not significantly different from the surviving fractions of clonal isolates irradiated with 5 or 10 Gy after surviving a previous exposure to 10 Gy (5 Gy, 0.065 ± 0.028; 10 Gy, 0.00103 ± 0.000552).

Chromosomal instability. The single copy of human chromosome 4 within the somatic cell hybrid was remarkably stable. In >4,000 metaphase cells analyzed from unirradiated populations, only two chromosomal rearrangements were observed. To assay for chromosomal instability, we analyzed 200 metaphase spreads from each clonal isolate for the presence of rearrangements, e.g., deletions, insertions, and reciprocal and nonreciprocal exchanges (translocations) involving the human chromosome. Clonal isolates expanded from a single surviving cell were said to be showing chromosomal instability when two or more metaphase cells were found with rearrangements involving chromosome 4. A rearranged population consisted of one or more metaphase cells showing the same rearrangement of the human chromosome. In irradiated cells, three of the six clonal isolates that survived the first exposure to 10 Gy (X2, X4, and X5) showed delayed chromosomal instability (Table 1). Analysis of 200 metaphase spreads from clone X4 indicated that 125 were normal, with no change involving human chromosome 4. There were five rearranged populations, consisting of 66, 5, 2, 1, and 1 metaphase spreads with the same rearrangement. Of the 200 metaphase cells analyzed from the X2 clone, 29 unique populations involving rearrangements of

TABLE 1. PE and metaphase populations with rearranged chromosome 4 in primary colony isolates

Clone	PE	% Normal metaphases[a]	No. of populations with rearrangements[b]
Unirradiated			
C1	0.69	100	0
C2	0.69	100	0
C3	0.83	100	0
C4	0.77	100	0
C5	0.59	100	0
Irradiated (10 Gy)			
X1	0.73	100	0
X2	0.35	1.5	29
X3	0.62	100	0
X4	0.30	62.5	5
X5	0.45	99	2
X6	0.78	100	0

[a] Percentage of metaphase cells showing no rearrangements of chromosome 4. Two hundred metaphase cells from each clone were analyzed.

[b] Number of metaphase spreads showing unique rearrangements of chromosome 4. A rearranged population consisted of one or more metaphase cells showing the same rearrangement(s) of the human chromosome.

chromosome 4 were observed. Examples of some of these populations are shown in Fig. 2. Ninety percent of metaphases analyzed from X2 showed the same two insertions of human DNA into hamster chromosomes (Fig. 2B, arrows). It was the rest of human chromosome 4 that showed delayed instability and contributed to the rearranged populations observed in this cell clone (Fig. 2B to G). Clonal isolates from X5 also showed distinct metaphase populations with rearranged chromosome 4 arising from a single cell (Table 1), again implicating delayed chromosomal instability. In addition, the PEs of subclones X2, X4, and X5 were reduced to 49, 42, and 63%, respectively, from the average PEs of the five unirradiated control clones (C1 to C5, average PE, 0.71) (Table 1).

The second round of subcloning indicates the longevity of this delayed instability (Table 2). Two subclones of the X2 clone, X2b[control] and X2c[control], continued to show reduced PE and new rearrangements of chromosome 4 in metaphase populations more than 22 generations beyond the initial DNA damage. Furthermore, the emergence of chromosome instability in the progeny of the X1 isolate was revealed only after the second round of subcloning. X1 was a colony surviving 10 Gy of X rays that, when initially expanded, showed no rearrangements involving chromosome 4 and had a normal PE (Table 1). An expanded subclone of this isolate (X1c[control]) revealed chromosome destabilization resulting in four distinct metaphase populations after subcloning (Table 2). One of these populations is shown in Fig. 3. Figure 3A shows five normal metaphase cells and a single metaphase cell with three rearranged hamster chromosomes showing translocations or insertions of the human chromosome. Fig. 3B shows another example of this destabilization of chromosome 4. Note also the grossly abnormal morphology of the interphase cell next to the metaphase chromosomes. It shows abnormal partitioning of the genetic material resulting in obvious micronucleus formation. This process usually results in the loss of genetic material in subsequent mitoses, leading to either aneuploidy or cell death.

Delayed chromosomal instability was a common event among the irradiated clones. Of the 14 colonies surviving a single dose of 5 Gy (C1a[5Gy] through C5c[5Gy]), 4 (29%) showed ≥two metaphase populations with rearranged chromosomes (Table 2). Of the 21 colonies surviving a single dose of 10 Gy (6 primary isolates, X1 through X6, and 15 secondary isolates, C1a[10Gy] through C5c[10Gy]), 13 (62%) showed ≥two metaphase populations with rearranged chromosomes (Tables 1 and 2). Subclones that were expanded from the primary colony isolates receiving 10 Gy (X1 through X6) and exposed to a second dose of 10 Gy (X1a[10Gy] through X6c[10Gy]) showed fewer instances of chromosomal instability than did the previously unirradiated isolates (C1a[10Gy] through C5c[10Gy]): Only 6 of the 19 (32%) subclones surviving two 10-Gy irradiations showed multiple rearrangements (Table 2).

Reduced PE. Our data show that colonies surviving X irradiation continue to have depressed PEs at least 44 generations after irradiation. The individual subclones showing markedly reduced PE often showed delayed chromosomal instability as well (see, for example, X2b[control] [Table 2]). Of the 25 primary and secondary colony isolates that demonstrated significantly depressed PE (PE ≤ 0.35; i.e., >2 standard deviations below the mean PE for the control colony isolates), 21 (84%) isolates also showed some kind of rearrangement involving chromosome 4. Furthermore, it is possible that the isolates with low PE but an ostensibly normal metaphase (one with no rearrangement of chromosome 4) could contain rearrangements exclusively among the hamster chromosomes. An example of cytogenetic changes that involved only the hamster chromosomes independent of the human chromosome is illustrated in Fig. 2C.

Aneuploidy. Aneuploidy is common in CHO cells and can be enhanced in populations after DNA damage (29). Although we primarily analyzed delayed chromosomal instability in pseudodiploid cells showing one copy of chromosome 4, we also examined the unirradiated primary colony isolate C4, which was tetraploid, with two intact copies of chromosome 4. Unirradiated subcolony isolates (C4a through C4c) showed metaphase populations that had gained or lost copies of chromosome 4. Tetraploid cell subclones surviving 5 or 10 Gy of X rays (C4a[5Gy] through C4c[10Gy]) seemed particularly susceptible to chromosome

FIG. 2. Examples of chromosomal rearrangements reflecting some of the metaphase populations observed in the primary colony isolate X2. In all figures, the human chromosome 4 is the yellow fluorescent chromosome in the background of red hamster chromosomes. (A) Metaphase cell showing a normal, nonrearranged chromosome 4. (B) The two standard (insertional) rearrangements observed in 90% of X2 metaphase populations are indicated with arrows. The rest of chromosome 4 is still relatively intact, with a terminal translocation and an interstitial insertion. (C to F) The two standard rearrangements are stable, but at the apparent site of the interstitial insertion shown in panel B is a range of chromosomal rearrangements involving various hamster chromosomes. (C) Translocation of the long arms of a hamster chromosome to chromosome 4. In this metaphase there is also a triradial involving only hamster chromosomes (arrow). (D) A dicentric. (E) No rearrangements involving hamster chromatin. (F) Formation of a new rearrangement at the centromeric site of chromosome 4 or, more probably, the loss of the centromeric region and the short arms of chromosome 4. (G) The most common metaphase population observed in X2. (H) The total loss of chromosome 4, except for the two standard rearrangements.

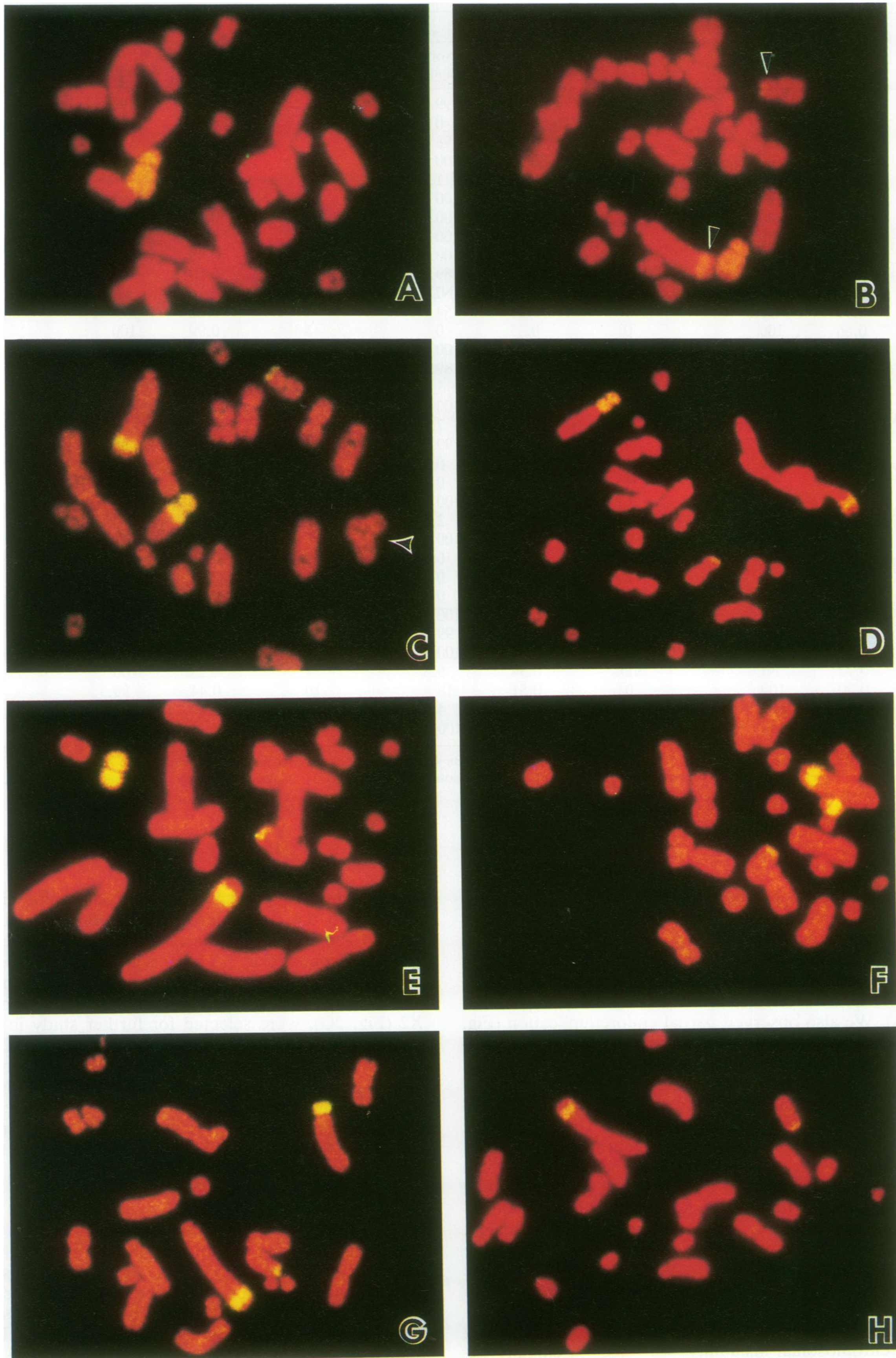

A
B
C
D
E
F
G
H

TABLE 2. PE and metaphase populations with rearranged chromosome 4 in secondary colony isolates

Subclone	Control			5 Gy			10 Gy		
	PE	% Normal metaphases[a]	No. of populations rearranged[b]	PE	% Normal metaphases	No. of populations rearranged	PE	% Normal methaphases	No. of populations rearranged
C1a	0.40	100[c]	0	0.46	99.5	1	0.25	99.5	1
C1b	1.01	100	0	0.48	0	1	0.54	100	0
C1c	0.99	100[c]	0	0.60	98	2	0.49	100	0
C2a	0.68	100	0	0.78	100	0	0.71	100	0
C2b	1.06	100	0	0.88	1.5	8	0.32	98.5	3
C2c	0.51	100	0	0.60	100	0	0.34	94	5
C3a	0.61	100	0	1.02	100	0	0.20	76	4
C3b	0.69	100	0	0.49	100	0	0.49	3.5	2
C3c	0.75	100	0	0.49	100	0	0.48	91	2
C4a	0.66	100	0	0.31	89.5	5	0.20	43.5	30
C4b	0.67	99.5	1	ND[d]	ND	ND	0.16	17.5	17
C4c	0.70	99.5	1	0.27	54	7	0.21	96	4
C5a	0.66	100	0	0.35	0	1	0.99	100	0
C5b	0.47	100	0	0.51	100	0	0.91	98.5	3
C5c	0.66	100	0	0.58	89.5	1	0.57	45	4
X1a	0.47	99.5	1	0.46	100	0	0.48	98.5	2
X1b	0.33	100	0	0.58	7.5	3	0.54	99.5	1
X1c	0.53	24	4	0.48	99.5	1	0.38	95	1
X2a	0.54	99.5	1	0.14	0	7	0.37	0	1
X2b	0.18	0	31	0.32	0	4	ND	0	2
X2c	0.43	6.5	6	0.62	100	0	0.42	0	3
X3a	0.77	100	0	0.59	2.5	3	0.54	0	2
X3b	0.76	100	0	0.59	100	0	0.45	1.5	3
X3c	0.84	100	0	0.70	0.5	2	0.40	99.5	1
X4a	0.29	0	2	0.24	0	1	0.39	99.5	1
X4b	0.58	19.5	1	0.29	0	1	0.23	0	1
X4c	0.29	0	3	0.22	100	0	0.40	100	0
X5a	0.40	100	0	0.48	99	1	ND	ND	ND
X5b	0.53	100	0	0.47	100	0	0.52	100	0
X5c	0.77	ND	ND	0.34	9.5	4	0.25	100	0
X6a	0.70	100	0	0.51	18	10	0.44	99.5	1
X6b	0.99	100	0	0.57	4.5	1	0.47	100	0
X6c	0.56	100	0	0.99	100	0	0.33	0	1

[a] Percentage of metaphase cells showing no rearrangements of chromosome 4. Two hundred metaphase cells from each clone were analyzed.

[b] Number of metaphase spreads showing unique rearrangements of chromosome 4. A rearranged population consisted of one or more metaphase cells showing the same rearrangement(s) of the human chromosome.

[c] Chromosome 4 lost from the population.

[d] ND, not determined.

rearrangement; all showed ≥four rearranged metaphase populations (Table 2).

There were increases in the frequency of tetraploid cells in all irradiated populations (data not shown). Tetraploidy may have resulted from failed cytokinesis or from endoreduplication. We saw a number of examples of endoreduplication (Fig. 4A). We also observed partial endoreduplication (Fig. 4B), which resulted in an asymmetric distribution of chromosomes in the resulting tetraploid cells (Fig. 4C and D). Although we did not analyze these tetraploid cells for chromosomal rearrangements, they also showed abnormalities involving chromosome 4 (Fig. 5). For example, from the X2 subcolony tetraploid cells with dicentric chromosomes (Fig. 5A, B, D, E, and F), abnormal segregation of the components of these broken chromosomes (Fig. 5B and C) and chromosomes with increased amounts of chromosome 4 (Fig. 5G) were observed. This implies chromosome fusion, breakage, and refusion as a potential mechanism for the observed chromosomal instability. Abnormal interphase cells showing evidence of unequal cytokinesis and micronuclei (Fig. 5H) were also observed.

SCE. When we realized that the X2 isolate was showing extreme chromosomal instability, we selected, clonally expanded, and froze a number of secondary colony isolates in addition to the X2a[control] to X2c[control] isolates described above. These isolates were later thawed and analyzed cytogenetically. Three subclones, two of which showed continuing instability involving chromosome 4 and one of which was the most common population observed in X2 (Fig. 2G), were selected for further study in a second cytogenetic assay for chromosome instability, SCE formation. No differences in SCE yields between the populations with rearranging chromosomes (X2c1 and X2c2), the population with stably rearranged chromosomes (X2c3), and a normal unirradiated control were found. SCE frequencies per chromosome were 0.34, 0.43, 0.40, and 0.42 for X2c1, X2c2, X2c3, and the control, respectively.

DISCUSSION

Although the human chromosome in the human-hamster hybrid cell line GM10115 is remarkably stable, a significant increase in the number of metaphase populations showing rearrangements of the human chromosome was seen after induction of DNA damage by ionizing radiation. Using the human chromosome as the basis for damage analysis proved

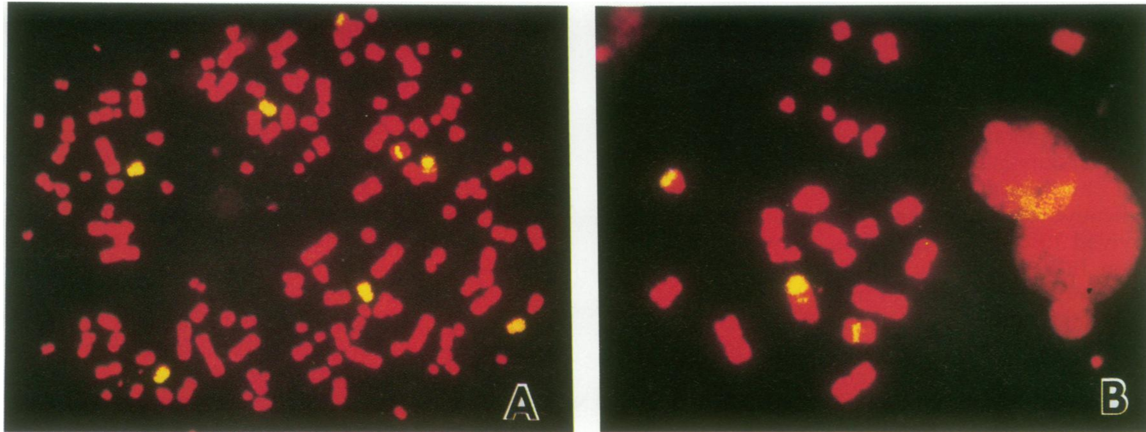


FIG. 3. Examples of chromosomal instability seen in the secondary colony isolate X1c[control]. (A) Six metaphase cells are shown: five have a normal, nonrearranged chromosome 4, and the other shows an example of delayed chromosomal instability. (B) Another example of the unstable metaphase population shown in panel A. Chromosome 4 is now rearranged within three hamster chromosomes. Two terminal translocations and two insertions of fragments of chromosome 4 can be observed.

to be an elegant and efficient system, but it must be noted that damage exclusive to the hamster chromosomes was also observed. Because the many populations of metaphases with rearranged chromosomes were seen in the progeny of isolates stemming from a single parent cell, it must be assumed that these rearrangements were not induced directly by the radiation exposure but occurred sometime after cell recovery and proliferation. This instability occurred soon after

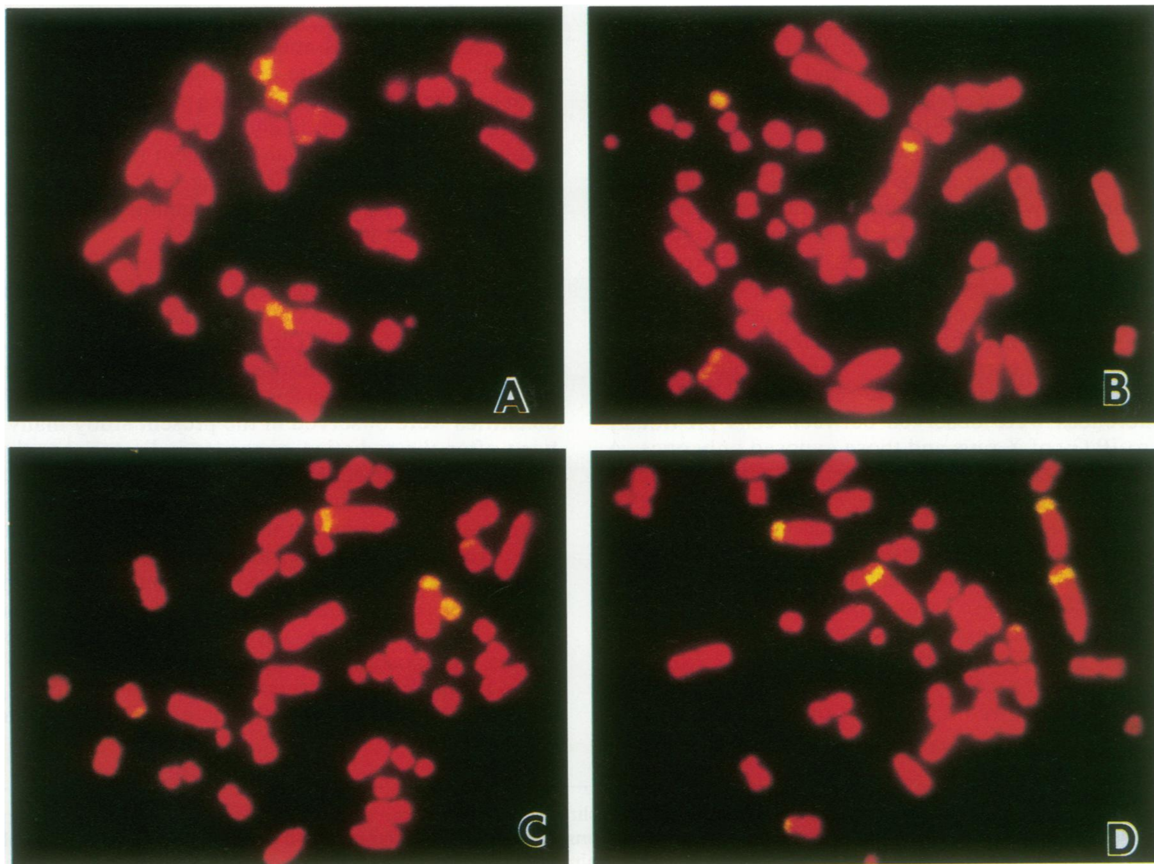


FIG. 4. Examples of pseudotetraploid metaphase populations seen in X2 cells. (A) Endoreduplicated cell showing two copies of the most common metaphase population observed in X2 (Fig. 2G). (B) Partially endoreduplicated cell showing abnormal segregation of the rearranged chromosome 4. Note the single copy of the large acrocentric chromosome with an insertion from human chromosome 4. (C) Pseudotetraploid cell showing two copies of some of the rearrangements involving chromosome 4 and only one copy of others. (D) Pseudotetraploid cell showing two copies of all the rearrangements involving chromosome 4 (Fig. 2G and panel A above).

irradiation in some surviving colonies (e.g., X2) and persisted in subclones of this original survivor for several generations (e.g., control subclones X2b and X2c). In other surviving colonies (e.g., X1), instability was not manifested for at least 22 generations, because multiple populations began to appear only in the second round of subcloning. The presence of chromosomal damage itself, however, was not always indicative of continuing instability. The clone X4, which survived 10 Gy of X radiation, showed a reciprocal translocation that was stably maintained in various subclones and showed no further chromosomal changes.

Our data indicate that delayed chromosomal instability occurs after exposure to ionizing radiation. This is in contrast to the initial observations of Kadhim et al. (23), who did not find evidence for chromosomal instability following X-ray exposure. The reason(s) for the discrepancy between the two studies is unclear. Kadhim et al. (23) looked only at chromosomal aberrations. Such rearrangements are generally transient and result in cell death. Fluorescence in situ hybridization is a more sensitive technique that can detect rearrangements accumulating over time and stably passed from generation to generation, as well as newly occurring rearrangements that may lead to cell death at the subsequent mitosis. Our observations are in agreement with a recent report by Holmberg et al. (20), who found clonal chromosomal aberrations and genomic instability in 65% of X-irradiated human T lymphocytes cultured for 9 to 13 days and analyzed cytogenetically by Giemsa banding. Chromosomal instability has also been observed 10 cell divisions after exposure of mouse hematopoietic cells to particles from plutonium-238 (23) and between 15 and 25 passages after exposure of primary human skin fibroblasts to the heavy ions neon, argone, or lead (26, 39).

Cells surviving an initial dose of 10 Gy of X rays were also subjected to a second round of X irradiation. The purpose of these experiments was to determine whether cells surviving a high dose of X rays (clones X1 to X6) were likely to be more sensitive or more refractory to a second round of irradiation as measured by PE and chromosomal rearrangements. The first 10-Gy dose was sufficient to induce a number of proteins that function in cellular responses to DNA damage, e.g., chromosomal repair, potentially lethal damage repair, and X-ray adaptive response (2, 3, 14, 49). Small differences were observed between cells previously exposed to 10 Gy of X rays and those exposed only once. A decrease in the frequency of chromosomal rearrangements was observed in cells expanded from colonies surviving two 10-Gy doses; when instability was observed in a particular clone, it could have been the product of the first dose of X rays. For further study of inducible or adaptive cellular pathways to DNA damage, it may be of interest to investigate the effects of damage with lower doses of X rays.

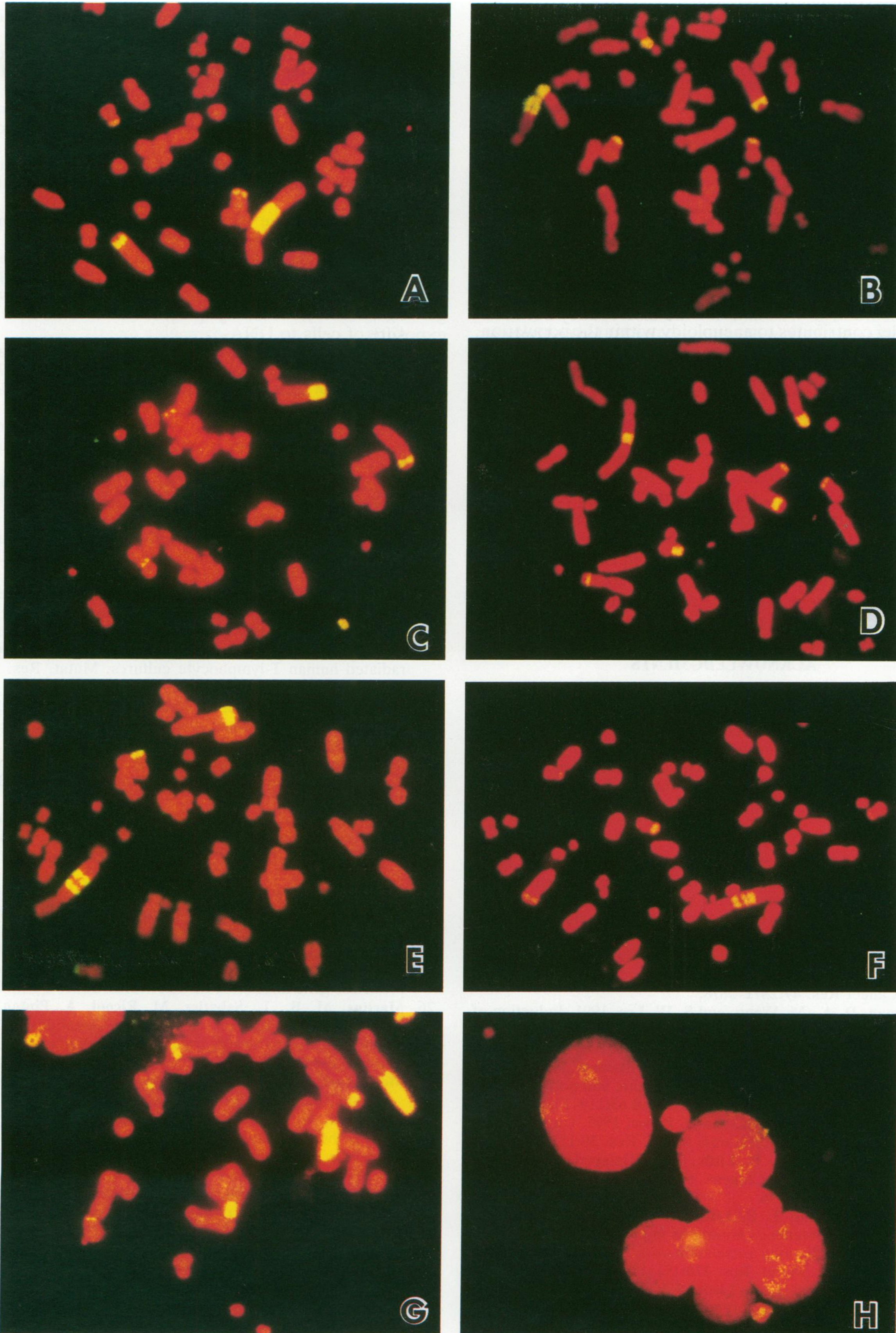
Twenty-one of 25 surviving colonies that exhibited delayed reproductive cell death (6–9), as manifested by reduced PE, also showed some kind of rearrangement involving chromosome 4. This implicates chromosome rearrangements as important contributors to the cells' inability to survive many generations after X-ray exposure. Since there appears to be no "dose response" of PE to the number of rearranged populations, the data suggest that the reduced PEs could also be modulated by factors other than mitotic cell death stemming from delayed chromosomal instability.

The effect of this delayed destabilization on SCE formation was also investigated. Although the observed induction of gross chromosome rearrangements in these clones did not influence the reciprocal exchange of homologous DNA sequences between sister chromatids, it is reasonable to assume that delayed chromosomal instability plays a significant role in gene mutation (7, 16, 32, 40), gene amplification (17, 18, 33, 38, 50), cellular transformation (26, 28), teratogenesis (15, 35), and even carcinogenesis (13, 30) after exposure of cells to DNA-damaging agents.

How ionizing radiations induce this delayed chromosomal instability is not known. Exposure of cells to ionizing radiation results in a variety of DNA lesions, including DNA base alterations, DNA-DNA and DNA-protein cross-links, and single- and double-strand DNA breaks (46). There is substantial evidence that the DNA double-strand break is the primary lesion involved in chromosomal rearrangements (37). On average, 40 double-strand breaks per gray of radiation are induced (47), implying that 200 and 400 breaks were induced in cells after 5 and 10 Gy of X rays, respectively. DNA double-strand breaks are rapidly rejoined after radiation exposure (22, 48) and are unlikely to contribute to the delayed chromosome instability observed here. It is possible that these late-appearing rearrangements result from a subset of persistent lesions passed from generation to generation. It is more likely, however, that the instability results from a radiation-induced event that deletes a gene or genes responsible for maintaining genomic integrity or by inducing a cellular process or endogenous virus that can lead to chromosomal instability.

Delayed chromosomal instability appears to result from two separate mechanisms. The first is related to the increase in the frequency of dicentric chromosomes observed here and by others (26, 39). Dicentric chromosomes may persist over a few cell generations but are generally considered unstable and not transmissible over time (5, 27). That dicentrics were observed in the present study many generations after the radiation exposure indicates that they were not a direct consequence of damage induced by ionizing radiation. Instead, dicentric formation probably arose from telomeric (39) or interstitial telomeric fusion. Interstitial telomere repeat sequences are common in the GM10115 cell line used here, as determined by in situ hybridization of a fluorescence-labeled telomeric probe (11). Although this probe does not indicate large interstitial telomeres in the human chromosome, the chromosomal distribution of telomeric repeat sequences is polymorphic in the human genome (4), and such repeat sequences have been proposed to be hot

FIG. 5. Examples of chromosome fusion resulting in a dicentric chromosome and some of the possible consequences of a fusion-bridge breakage-refusion cycle in tetraploid cells from X2. (A) Dicentric chromosome involving a large region of chromosome 4. (B) Possible results of a bridge-breakage event resulting in two chromosomes with terminal regions containing chromosome 4. (C) Possible consequences of random segregation of chromosome regions after bridge breakage. Comparing panels B and C gives an idea of the asymmetric segregation of chromosomes after bridge breakage. (D) Formation of a dicentric chromosome involving part of chromosome 4; however, note the newly evolving chromosomes containing chromosome 4 predominantly as terminal translocations. (E and F) Dicentric chromosomes resulting from the fusion of human and hamster chromatin. (G) Probable results of bridge breakage, i.e., the abnormal accumulation of large regions of chromosome 4 in certain cells. (H) Abnormal attempts at cell division. Note the two large micronuclei, one containing part of chromosome 4.


A
B
C
D
E
F
G
H

spots for recombination, breakage, and fragility (19, 33). Dicentric chromosomes provide the opportunity for chromosome breakage at anaphase, and subsequent refusion of these broken chromosomes initiates the fusion-bridge breakage-refusion cycle. Breakage resulting in the formation of dicentrics could lead to both delayed and continued instability. Dicentric formation has long been considered a mechanism for another endpoint of genetic instability, gene amplification (24, 38, 42, 45). Dicentrics that are formed during gene amplification can initiate another wave of chromosomal instability (25, 38). The second possible mechanism for delayed chromosomal instability is related to the delayed induction of micronuclei. Micronuclei can be formed as a consequence of chromosome breakage, e.g., terminal and/or interstitial deletions resulting in acentric chromosome fragments (10), or as a result of spindle dysfunction (34). Loss of micronuclei contributes to aneuploidy within the population, a phenomenon commonly observed after exposure to ionizing radiation (8) and during gene amplification (45).

The delayed chromosomal instability described here can directly affect a number of genetic changes that make up the progressive stages leading to cancer. Indeed, the presence of a myriad of chromosomal rearrangements in preneoplastic and neoplastic cells indicates the role of genomic instability in the neoplastic process. Ionizing radiation can result in the transformation of a cell from the normal to the neoplastic state both in vivo and in vitro. The high frequency with which X rays induce this delayed chromosomal instability makes this an ideal system with which to investigate the mechanisms of chromosome rearrangements and the consequences of chromosome destabilization.

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